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A structural model for the glutamate-specific endopeptidase from *Streptomyces griseus* that explains substrate specificity

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We present a model for the three-dimensional structure of the glutamate-specific endopeptidase from *Streptomyces griseus* based on the crystal structures of other bacterial proteases of the trypsin family. For the first time a structural model is described which attempts to explain the basis of P1 glutamate specificity in serine proteases. Several important changes to the S1 pocket with respect to other members of the family of different specificity are described. Of particular interest is the presence of a histidine at position 213 and the substitution of Arg-138 by lysine. Other biochemical evidence concerning substrate preferences can be rationalized on the basis of the model.

Glutamate-specific endopeptidase; Serine protease; Substrate specificity; S1 binding pocket; *Streptomyces griseus*

1. INTRODUCTION

Glutamate-specific endopeptidases (GSEs) have been widely utilized in protein sequencing due to their highly selective substrate specificity [1]. The most widely studied and best characterized of these enzymes is that from *Staphylococcus aureus* strain V8 (V8 protease) originally described by Drapeau et al. [2]. More recently several other GSEs from different species have been reported, together with complete or partial amino acid sequences. The complete sequence of the GSE from *Bacillus licheniformis* shows 25% identity to the V8 protease [3] and those from *Actinomyces* spp. [4] and *Streptomyces thermovulgaris* [5] are both similar to V8 at their N-termini, suggestive of an homologous relationship. A glutamate specific endopeptidase isolated from *Streptomyces griseus*, however, was reported to be unrelated to the V8-like family, but instead to show considerable similarity to proteases A and B from *S. griseus* itself (SGPA and SGPB), with which it shares 59% and 56% identity, respectively, and to α-lytic (α-LP) protease from *Lysobacter enzymogenes* (36% identity) [6].

All GSEs show a marked preference for substrate cleavage on the C-terminal side of glutamic acid residues (i.e. with Glu in the P1 position [7]). In the case of V8 protease such substrates are hydrolized up to 5,000-times faster than substrates with P1 aspartic acid [8]. Furthermore, the specificity of these enzymes appears to be largely determined by their S1 subsites alone [1].

The above mentioned GSEs appear to be serine proteases. In the case of the enzyme from *S. griseus* (SGPE) this is apparent from its clear homology to other members of the trypsin family, and in the case of V8 (and, by implication, the remaining enzymes) due to reactivity of a specific serine residue with diisopropylfluorophosphate [9]. Furthermore, it has been suggested that the V8 protease itself is a distant relation of the trypsin family [9,10], implying a common origin and structural fold, similar to that of trypsin, for all GSEs thus far identified.

The three-dimensional structures of several members of the trypsin family of both mammalian and bacterial origin, and in both the presence and absence of inhibitors, are known. Such knowledge has proved invaluable in contributing to an understanding of substrate specificity. For example, the presence of Asp-189 deep in the S1 subsite of trypsin [11], thrombin [12], kallikrein [13] and tonin [14], readily explains their specificity for P1 arginine or lysine. On the other hand, the substitution of Asp-189 in chymotrypsin [15] and rat mast cell protease II [16], and by a somewhat rearranged S1 pocket in proteases A and B from *S. griseus* [17,18], render these enzymes specific for large hydrophobic residues at P1. In elastase [19], substitutions at positions 216 and 226, and in α-LP [20] at 190 and 213, restrict the size of the S1 pocket to allow only small hydrophobic side chains. In the case of elastase, model building experiments [21] were successful in the recognition and understanding of substrate specificity prior to the determination of the crystal structure [22]. Furthermore, in

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Abbreviations. SGPA, SGPB, SGPE, *Streptomyces griseus* proteases A, B and E; α-LP, alpha-lytic protease; GSE, glutamate-specific endopeptidase

the studies of chymotrypsin and trypsin, it was the very knowledge of the substrate specificities that aided in the correction of errors in the sequence, thus permitting the recognition of the primary binding site and 'charge-relay system' [21,23].

In comparison with the plethora of structural information concerning enzymes with trypsin-like, chymotrypsin-like or elastase-like specificity, the structural basis behind GSE specificity remains somewhat poorly understood and represents a considerable gap in our knowledge. Attempts to modify trypsin by substitution of Asp-189 by a lysine did not produce an acid-specific enzyme but rather a preference for Phe/Tyr/Leu at P1 [24]. In the absence of an experimentally determined structure, we have constructed a model for SGPE based principally on SGPA in an attempt to shed light on its substrate specificity. SGPE was chosen from the group of GSEs of known sequence on the basis of its high degree of sequence identity with SGPA and SGPB and to a lesser extent with α-LP, which should not only ease the modelling process but increase the reliability of the results and the conclusions drawn from it [25]. The validity and utility of the methodology is exemplified by the successful modelling of the elastase structure [21] and the recent prediction of the substrate specificities of two granzymes from cytotoxic T lymphocytes which have subsequently been verified experimentally [26,27].

2. MATERIALS AND METHODS

The sequence alignment used for the molecular modelling of SGPE was based on that derived from structural equivalences by Fujinaga et al [20] modified to include SGPE [6]. Following convention, the chymotrypsinogen numbering [28] has been adopted throughout.

The backbone conformation for SGPE was based largely on that of SGPA with which it shares the greatest sequence identity. The loop in the region of residue 70, which required an insertion of seven residues, was built partially on SGPB and joined using the REFI option in the graphics program TOM [29] The region of residues 110–120 and the deletion at 173 were found to be modelled more satisfactorily when based on α-LP It was also necessary to change the backbone conformation in the region of Cys-222 in order to accommodate a deletion prior to the ½-cystine, an insertion after it and to remove steric clashes introduced by the substitution of glycines at 216 and 222C. The only other significant alterations to the main chain that were required were small adjustments in the region of Lys-156 and Ser-190.

The side chains for the 82 residue differences (substitutions and insertions) between SGPA and SGPE were replaced using TOM, and in cases where an identical amino acid was present in one of the remaining structures (SGPB or α-LP) their χ angles were adjusted in order to copy the side chain conformation Any remaining atomic clashes were removed with the DEBUMP option of the program WHATIF [30]. The model was energy minimized using 25 cycles of steepest decent, followed by conjugate gradient minimization using the GROMOS option of the program WHATIF [30].

The S1 pocket was modelled in the presence of a glutamic acid residue. For this purpose the structure of SGPA (Brookhaven code 5SGA), which has been solved in the presence of Ac-Pro-Ala-Pro-Tyr-OH [18], was used. The P1 tyrosine of the inhibitor was substituted for glutamic acid, and its side chain torsion angles, together with component residues of the specificity pocket, adjusted to give the best fit.

The quality of the resulting model was assessed using three methods. The program PROCHECK [31,32] evaluates the stereochemical quality of the structure whilst VERIFY3D [33] and the QUALITY CONTROL option of WHATIF [30] evaluate the chemical reasonableness and self-consistency of the model on a residue-by-residue basis by assessing local environments.

3. RESULTS AND DISCUSSION

Fig. 1 shows a C_α trace of the final SGPE model superposed on to that of SGPA. As anticipated, significant changes to the main chain are largely restricted to surface loops. Of these the most important is in the region of the disulphide bridge which unites ½-cystines 189 and 220. Due to the deletion of one residue prior to Cys-220 and an insertion of one residue following, it the conformation of this region required considerable alteration. Furthermore, the substitution of Gly-216 by serine (which resulted in severe steric hindrance) and the loss of Gly-222C, the main chain torsion angles of which lie outside the permitted region, support the notion that this region of the molecule differs in SGPE. Its conformation therefore probably represents one of the least well determined parts of the structure.

Overall, however, the model is of high quality, as judged by stereochemical criteria and by its chemical self-consistency. All of the stereochemical analyses of the PROCHECK program (Ramachandran distribution, C_α chirality, peptide bond planarity, side chain conformation, non-bonded contacts, etc.) show the model to be within the limits expected of a structure at 1.8 Å resolution (that of SGPA) and are generally superior. Residue environment analysis by the method of Luthy et al. [33] gave a total score of 91.21, close to that expected for a protein of 188 residues, and the mean value of the 21-residue moving window profile was 0.47. The QUALITY CONTROL option of WHATIF gave a mean value of −0.71 for the model which lies within the range expected for well-determined structures.

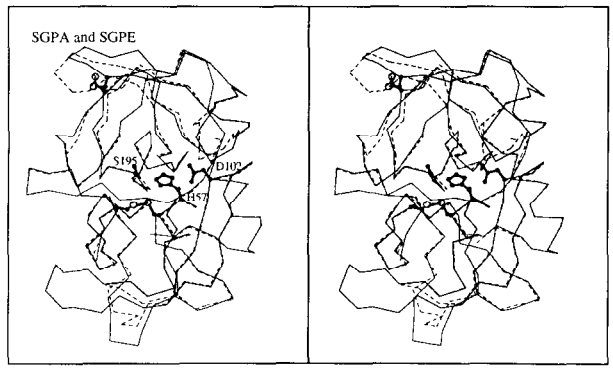

Fig. 1. Stereo C_α trace of the final SGPE model (solid line) superposed on to the crystal structure of SGPA. The molecule is seen looking down into the active site cleft, and the members of the catalytic triad are indicated The most significant changes are to the mainchain affect surface loops, particularly around the disulphide bridge between ½-cystines 189 and 220 (shown at the top of the figure). The drawing was produced with the program MOLSCRIPT [36].

Table I

Some of the most significant residue substitutions observed in SGPE with respect to SGPA/SGPB and α-LP, together with a description of the normal structural role of these residues

Residue	Substitution	Normal role
138	R→K	Salt-bridge with Asp-194
156	G→K	Carbonyl stabilizes Arg-138 ϕ,ψ in disallowed region
190	A/M→S	Forms part of S1 pocket
213	T/M→H	Forms part of S1 pocket
216	G→S	Forms part of S1 pocket and extended binding site

The members of the catalytic triad are conserved in SGPE, as expected for an active protease. Furthermore, Ser-214, sometimes described as the fourth member of a catalytic *tetrad* [19,34] is also conserved and forms the expected hydrogen bond with Asp-102 within the active site cleft. The most significant residue changes with respect to the other bacterial sequences shown in Fig. 2 are given in Table I. The importance of these substitutions is emphasized by the fact that they are also observed in a closely related GSE from *S. fradiae*, the sequence of which has been recently deposited [35]. Most of these changes affect or may affect the nature of the S1 pocket either directly or indirectly and are now described in detail.

The most noticeable feature of the S1 pocket in comparison with other members of the trypsin family is the presence of His-213. Histidine is conserved in the equivalent position in the V8 protease and in the GSE from *B. licheniformis*, and is thus probably characteristic of GSEs in general (unpublished results). Fig. 3 compares the S1 pocket of SGPE with that of trypsin and shows that the $N_{\delta 1}$ of His-213 forms one of several hydrogen bonds to the P1 glutamic acid. The outstanding hydrogen bonds come from the alcohol groups of Ser-190 and Ser-216 which (with the exception of Ser-190 in chymotrypsin and trypsin) are unique to SGPE (see Fig. 2). The position of Ser-190 is somewhat further removed from the S1 pocket than the equivalent residue in SGPA. This slight opening of the pocket is permitted by the presence of the smaller alanine at 188 (in place of valine), as also observed in α-LP. It is of note that the S1 pocket is very different from that of trypsin, and that the difference between the two enzymes is not simply an exchange of Asp-189 of trypsin for a basic amino acid in order to produce a complimentary change of substrate specificity. This is to be expected in the light of the mutagenesis experiments of Graf et al. [24], which suggest that a lysine at 189 may bury its positive charge in an internal hydrophilic pocket rather than point into the S1 subsite.

At the pH optimum of 9.0 [1] there seems little justification for assuming that His-213 would be protonated, and therefore would not be expected to supply a formal positive charge to neutralize that of the P1 glutamic acid on the substrate. The only other possible source of such a charge is Lys-138. In SGPA, SGPB and α-LP, an arginine in this position is involved in the formation of an internal salt-bridge with Asp-194. Stabilization of the buried Asp-194, however, is not achieved solely via interaction with Arg-138. Four strong hydrogen bonds stabilize the carboxylate group, and none of these are made directly with the arginine. The salt-bridge is instead mediated by the O_γ of Thr-143, which together with Thr-142 is involved in a network of hydrogen bonds and which is conserved in all of the bacterial structures. It is somewhat unexpected that Arg-138 should not be conserved in SGPE and this is suggestive of an alternative role.

We cannot rule out the possibility that this lysine simply performs an analogous function to that of the arginine normally found at this position, however, the absence of another candidate to provide a formal positive charge to the S1 pocket, and the very fact that it has been substituted, led us to investigate a second conformation. We have therefore modelled this lysine such that its side chain nitrogen enters more directly into the S1 pocket. The length of the lysine side chain, even in its fully extended conformation, does not permit direct interaction with the P1 glutamate, however, such an interaction is possibly mediated via Ser-190 in an analogous manner to that described above for the salt-bridge normally encountered between Arg-138 and Asp-194. In the presence of the substrate, the formal charge on the lysine would lie between Asp-194 and the P1 glutamic acid. A corollary of such a model is that the carbonyl of Gly-156, which normally aids in stabilizing the guanidinium group of Arg-138, would become free, releaving the obligate requirement for glycine at this position. In SGPE, Gly-156 is substituted by lysine, and the main chain in this region has been remodelled so as to eliminate the disallowed main chain torsion angles associated with the glycine.

An alternative model, in which Lys-156 forms the salt-bridge with Asp-194, was also considered. This would allow Lys-138 to participate solely in substrate binding. No suitable loop for the region encompassing Lys-156 could be found with the DGLP option of TOM which did not disrupt the residues involved in the stabilization of Asp-194. This possibility thus seems less attractive than that already described.

Besides the dominant P1 glutamate specificity, several other aspects of substrate preferences of SGPE can now be explained in terms of the structural model. Amongst those substrates tested by Breddam and Meldel [1], the least acceptable residue at P2 was found to be aspartic acid. Examination of the model shows that the substitution of Ser-174 in SGPA by aspartic acid in SGPE affects the nature of the base of the S2 subsite, rendering the binding of substrates with P2 acidic residues unfavourable. Thus the observed and unexpected cleavage of the substrate, ABz-Ala-Ala-

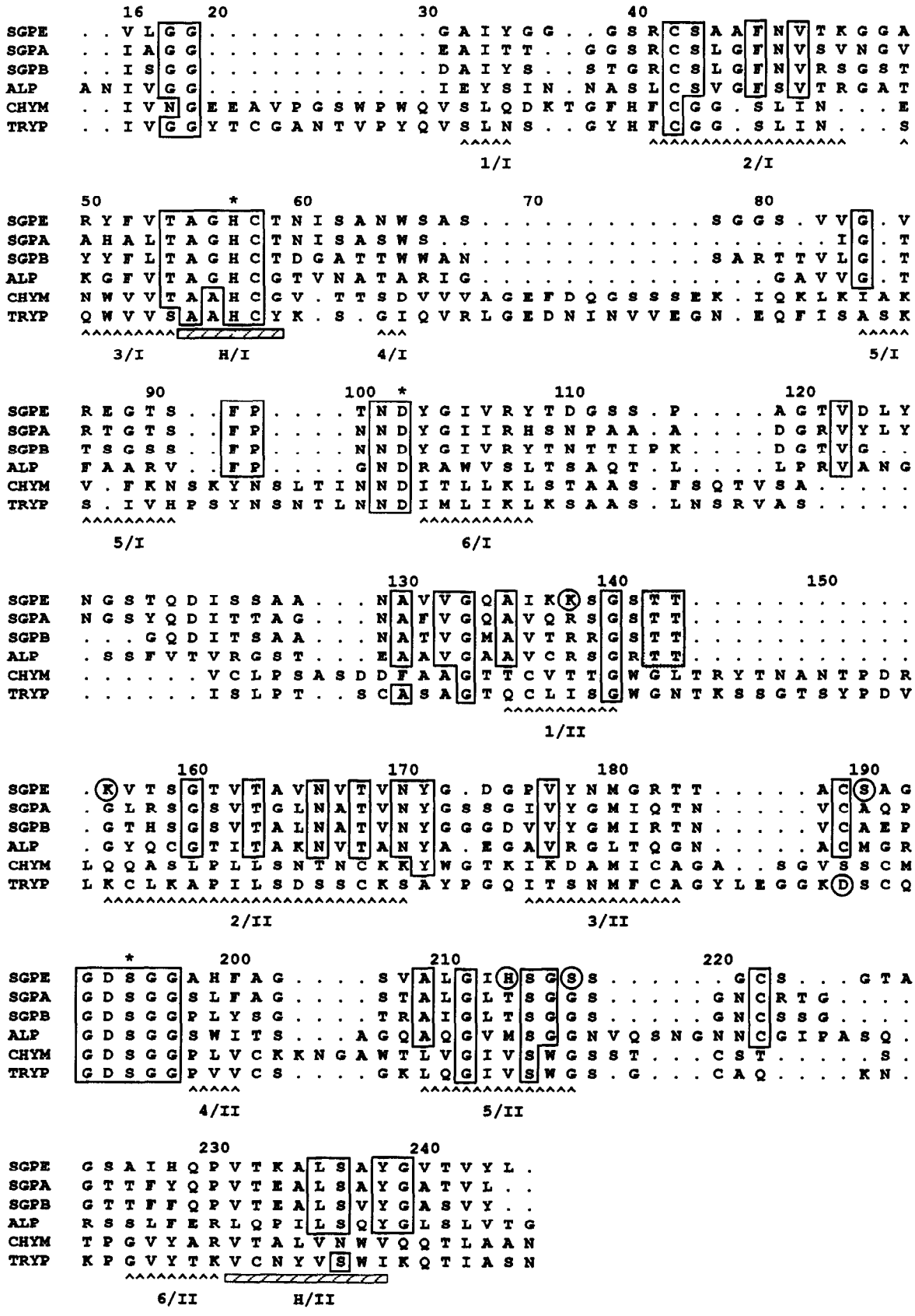
16 20 30 40
SGPE . . V L G G . . . . . G A I Y G G . . G S R C S A A F N V T K G G A
SGPA . . I A G G . . . . . E A I T T . . G G S R C S L G F N V S V N G V
SGPB . . I S G G . . . . . D A I Y S . . S T G R C S L G F N V R S G S T
ALP A N I V G G . . . . . I E Y S I N . N A S L C S V G F S V T R G A T
CHYM . . I V N G E E A V P G S W P W Q V S L Q D K T G F H F C G G . S L I N . . . E
TRYP . . I V G G Y T C G A N T V P Y Q V S L N S . . G Y H F C G G . S L I N . . . S
1/I 2/I
50 * 60 70 80
SGPE R Y F V T A G H C T N I S A N W S A S . . . . . S G G S . V V G . V
SGPA A H A L T A G H C T N I S A S W S . . . . . I G . T
SGPB Y Y F L T A G H C T D G A T T W W A N . . . . . S A R T T V L G . T
ALP K G F V T A G H C G T V N A T A R I G . . . . . G A V V G . T
CHYM N W V V T A A H C G V . T T S D V V V A G E F D Q G S S S E K . I Q K L K I A K
TRYP Q W V V S A A H C Y K . S . G I Q V R L G E D N I N V V E G N . E Q F I S A S K
3/I H/I 4/I 5/I
90 100 * 110 120
SGPE R E G T S . . F P . . . . T N D Y G I V R Y T D G S S . P . . . . A G T V D L Y
SGPA R T G T S . . F P . . . . N N D Y G I I R H S N P A A . A . . . . D G R V Y L Y
SGPB T S G S S . . F P . . . . N N D Y G I V R Y T N T T I P K . . . . D G T V G . .
ALP F A A R V . . F P . . . . G N D R A W V S L T S A Q T . L . . . . L P R V A N G
CHYM V . F K N S K Y N S L T I N N D I T L L K L S T A A S . F S Q T V S A . . . . .
TRYP S . I V H P S Y N S N T L N N D I M L I K L K S A A S . L N S R V A S . . . . .
5/I 6/I
130 140 150
SGPE N G S T Q D I S S A A . . . N A V V G Q A I K K S G S T T . . . . . .
SGPA N G S Y Q D I T T A G . . . N A F V G Q A V Q R S G S T T . . . . . .
SGPB . . . G Q D I T S A A . . . N A T V G M A V T R R G S T T . . . . . .
ALP . S S F V T V R G S T . . . E A A V G A A V C R S G R T T . . . . . .
CHYM . . . . . V C L P S A S D D F A A G T T C V T T G W G L T R Y T N A N T P D R
TRYP . . . . . I S L P T . . S C A S A G T Q C L I S G W G N T K S S G T S Y P D V
1/II
160 170 180 190
SGPE . K V T S G T V T A V N V T V N Y G . D G P V Y N M G R T T . . . . . A C S A G
SGPA . G L R S G S V T G L N A T V N Y G S S G I V Y G M I Q T N . . . . . V C A Q P
SGPB . G T H S G S V T A L N A T V N Y G G G D V V Y G M I R T N . . . . . V C A E P
ALP . G Y Q C G T I T A K N V T A N Y A . E G A V R G L T Q G N . . . . . A C M G R
CHYM L Q Q A S L P L L S N T N C K K Y W G T K I K D A M I C A G A . . S G V S S C M
TRYP L K C L K A P I L S D S S C K S A Y P G Q I T S N M F C A G Y L E G G K D S C Q
2/II 3/II
* 200 210 220
SGPE G D S G G A H F A G . . . . S V A L G I H S G S S . . . . . G C S . . . G T A
SGPA G D S G G S L F A G . . . . S T A L G L T S G G S . . . . . G N C R T G . . . .
SGPB G D S G G P L Y S G . . . . T R A I G L T S G G S . . . . . G N C S S G . . . .
ALP G D S G G S W I T S . . . A G Q A Q G V M S G G N V Q S N G N N C G I P A S Q .
CHYM G D S G G P L V C K K N G A W T L V G I V S W G S S T . . . C S T . . . . . S .
TRYP G D S G G P V V C S . . . . G K L Q G I V S W G S . G . . . C A Q . . . . K N .
4/II 5/II
230 240
SGPE G S A I H Q P V T K A L S A Y G V T V Y L .
SGPA G T T F Y Q P V T E A L S A Y G A T V L . .
SGPB G T T F F Q P V T E A L S V Y G A S V Y . .
ALP R S S L F E R L Q P I L S Q Y G L S L V T G
CHYM T P G V Y A R V T A L V N W V Q Q T L A A N
TRYP K P G V Y T K V C N Y V S W I K Q T I A S N
6/II H/II

Fig. 2. Sequence alignment of proteases E, A and B from *S griseus* (SGPE, SGPA, SGPB), α-lytic protease from *Lysobacter enzymogenes* (ALP), chymotrypsin (CHYM) and trypsin (TRYP). The numbering is that of chymotrypsinogen [28]. Members of the catalytic triad are marked by asterisks. Identical residues in the four bacterial sequences are boxed, and where these are also conserved in the mammalian proteins the box is extended to include these residues. Substitutions important for the structure of the S1 subsite in SGPE are marked by circles and described in Table I. The two conserved threonines important for the stabilization of the structure around Asp-194 are highlighted, and Asp-189, the substrate specificity-determining residue in trypsin, is indicated by a circle. The β-strands which form the two six-stranded barrels are indicated by the arrowhead symbol, and the two major helical regions by the hatched boxes. Strands and helices are labeled to indicate their sequential position (arabic numerals) and the domain to which they belong (roman numerals).

←

Glu-Glu-Tyr-NO_2-Asp-OH, between the two glutamates, as well as at Glu-Tyr-NO_2 [1], may be due to the reduced acceptibility of glutamic acid at P2 in comparison with GSEs from other sources.

Proline is disfavoured at P3 [1], probably because the absence of a free amide interferes with binding to the extended substrate binding site, in particular preventing the formation of the hydrogen bond to the carbonyl of Ser-216. At P1′, arginine was the best residue from among those examined, perhaps because of the size of the S1′ pocket and the possibility of an ion-pair formation with Asp-174 assuming an extended conformation for the arginine.

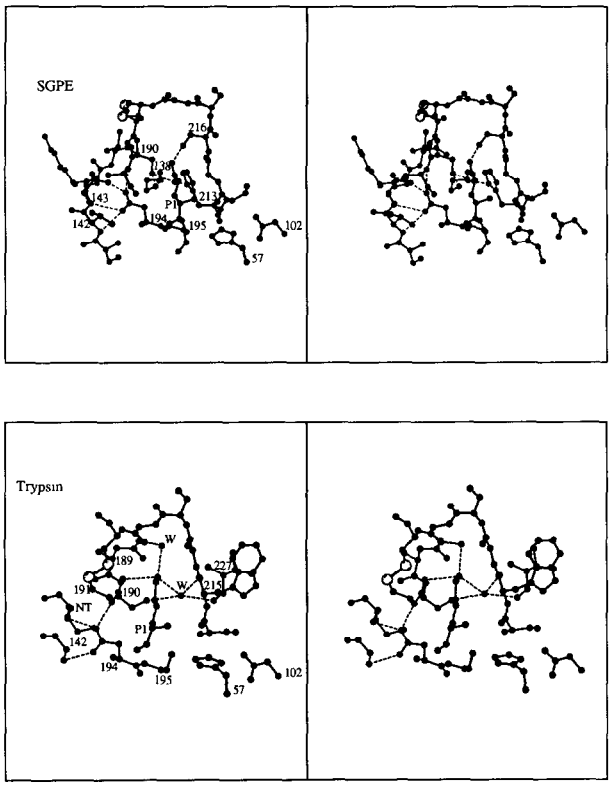

Fig. 3. Structure of the S1 pocket in SGPE compared with that in trypsin. (Upper panel) Modelled structure for SGPE with P1 glutamic acid. The substrate side chain is stabilized by hydrogen bonds to His-213, Ser-216, and via a salt-bridge to Lys-138 mediated by Ser-190. (Lower panel) Trypsin with P1 lysine, taken from the crystal structure of the trypsin–bovine pancreatic trypsin inhibitor complex [37]. Specificity for basic residues is principally determined by Asp-189 at the base of the S1 pocket, which, in the case of P1 lysine residues, interacts via a water molecule. The drawing was produced with the program MOLSCRIPT [36].

4. CONCLUSION

We have proposed a model for the glutamate specific endopeptidase of *S. griseus* which succeeds in identifying several apparently important substitutions with respect to other serine proteases, in particular in the region of the S1 pocket. The model accounts for the glutamate specificity by the arrangement of several of these residues in such a way as to permit the formation of several hydrogen bonds (His-213 seems to be of particular importance in this respect), and by the presence of Lys-138, the positively charged nitrogen of which enters the S1 pocket. Aspartic acid would be expected to fit less elegantly into the S1 pocket due to its shorter side chain.

The model suggest several mutagenesis experiments which might be used to further clarify the nature of substrate binding to the enzyme. Of particular interest would be the removal of His-213, perhaps by substitution with a valine, as found in chymotrypsin. Changing Lys-138 back to an arginine, or modifying its chemical nature altogether, might help to delineate its role more precisely.

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